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Mini-Review

# Role of Liquid–Liquid Separation in Endocrine and Living Cells

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**Abbreviations:** AR, androgen receptor; CBX2, chromobox 2; DSD, disorders of sex development; FUS, fused in sarcoma; IAPP, islet amyloid polypeptide; IDR, intrinsically disordered region; LLPS, liquid–liquid phase separation; MAMLD1, mastermind-like domain containing 1; NONO, non-POU domain containing octamer binding; PML, promyelocytic leukemia; SOX9, SRY-box transcription factor.

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## Abstract

**Context:** Recent studies have revealed that every eukaryotic cell contains several membraneless organelles created via liquid–liquid phase separation (LLPS). LLPS is a physical phenomenon that transiently compartmentalizes the subcellular space and thereby facilitates various biological reactions. LLPS is indispensable for cellular functions; however, dysregulated LLPS has the potential to cause irreversible protein aggregation leading to degenerative disorders. To date, there is no systematic review on the role of LLPS in endocrinology.

**Evidence acquisition:** We explored previous studies which addressed roles of LLPS in living cells, particularly from the viewpoint of endocrinology. To this end, we screened relevant literature in PubMed published between 2009 and 2021 using LLPS-associated keywords including “membraneless organelle,” “phase transition,” and “intrinsically disordered,” and endocrinological keywords such as “hormone,” “ovary,” “androgen,” and “diabetes.” We also referred to the articles in the reference lists of identified papers.

**Evidence synthesis:** Based on 67 articles selected from 449 papers, we provided a concise overview of the current understanding of LLPS in living cells. Then, we summarized recent articles documenting the physiological or pathological roles of LLPS in endocrine cells.

**Conclusions:** The discovery of LLPS in cells has resulted in a paradigm shift in molecular biology. Recent studies indicate that LLPS contributes to male sex development by providing a functional platform for SOX9 and CBX2 in testicular cells. In addition, dysregulated LLPS has been implicated in aberrant protein aggregation in pancreatic  $\beta$ -cells, leading to type 2 diabetes. Still, we are just beginning to understand the significance of LLPS in endocrine cells.

**Key Words:** cellular body, phase transition, paraspeckle, sex development, type 2 diabetes

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Eukaryotic cells contain not only classic membrane-bound organelles, such as the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus, but also several membraneless cellular bodies [1]. Recent studies have revealed that these membraneless organelles are created via a reversible physical phenomenon referred to as liquid-liquid phase separation (LLPS) [2–11]. During LLPS, liquids demix into the dense and light phases, as oil in water [12]. LLPS assembles specific sets of proteins and nucleic acids, thereby temporarily subdividing the intracellular space into functional compartments [13]. Subcellular compartmentalization by LLPS enables parallel progression of multiple biochemical reactions in a single cell [12].

To date, several types of LLPS-mediated organelles have been identified in eukaryotic cells, yet the list may still be incomplete [1, 10, 11, 14–17]. These organelles were implicated in various organic processes including transcriptional regulation and RNA processing [1, 4, 12–14, 16, 18–22]. On the other hand, dysregulated LLPS has the potential to produce irreversible protein aggregation and resultant cellular dysfunction [19]. The discovery of LLPS in living cells has caused a paradigm shift in molecular biology. This mini-review aims to provide a brief overview of the current understanding of LLPS in living cells and to discuss the possible roles of LLPS in health conditions, particularly in endocrinology.

## Methods and Results of Literature Search

We searched for relevant literature in PubMed (<https://pubmed.ncbi.nlm.nih.gov>) using various LLPS-associated keywords including “liquid-liquid phase separation,” “phase transition,” “paraspeckle,” “membraneless organelle,” and “intrinsically disordered,” combined with endocrinological keywords such as “hormone,” “endocrine,” “ovary,” “androgen,” “diabetes,” “sex differentiation,” “insulin,” and “SOX9.” We focused on articles published between 2009 and 2021. We also referred to the articles in the reference lists of identified papers.

As a result, we identified 449 articles. Of these, we selected 67 papers that provide important clues to understand the pathophysiological roles of LLPS in endocrine cells. We did not find any papers that describe the association between LLPS and autoimmune endocrine disorders.

## LLPS in Living Cells

### Discovery of LLPS in Cells

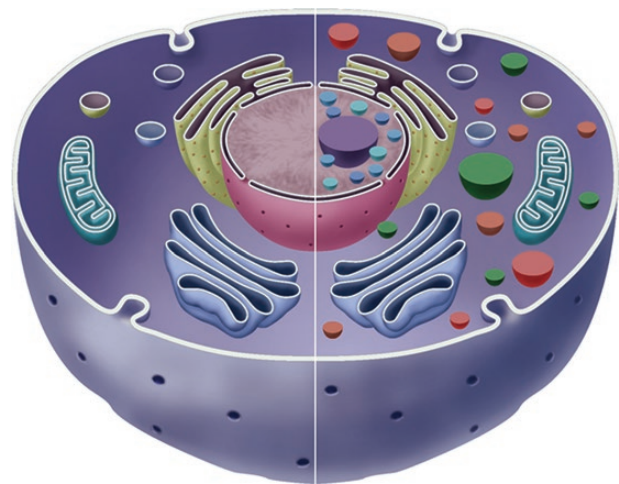
It has long been known that living eukaryotic cells contain many organelles that lack lipid layers [1]; however, the origin and contents of such membraneless cellular bodies

remained unknown until recently. In 2009, Brangwynne et al. reported liquid-like behaviors of P granules, a membraneless organelle detected in *Caenorhabditis elegans* [10]. The authors found that P granules rapidly dissolved and condensed in the germ cells. Subsequent studies revealed that LLPS, a well-studied phenomenon in soft matter physics, is involved in the formation of P granules and other membraneless organelles in eukaryotic cells [2, 3, 11, 23]. LLPS generates a phase boundary in the subcellular space to confine functional entities [12, 14]. LLPS represents a reversible phase transition that is spatiotemporally regulated by intra- and extracellular conditions [15].

LLPS creates various types of cellular bodies, each of which encompasses a unique set of proteins and nucleic acids and facilitates specific biological reactions [14]. These organelles can exchange their components with the surrounding environment and can also interact with classic membrane-bound organelles [24, 25]. Organelles created by LLPS usually persist in the cell for hours to days, but can turn over their contents within seconds to minutes [1]. Such a highly dynamic nature of membraneless organelles differs from the relatively long lifecycle of classic membrane-bound organelles [26]. Indeed, unlike LLPS-mediated cellular bodies, classic organelles rarely originate de novo [26].

## Cellular Bodies Created by LLPS

Every eukaryotic cell encompasses a number of membraneless organelles formed via LLPS (Fig. 1) [1].



**Figure 1.** The image of organelles in a human cell. The left half of this figure shows a schematic of classic membrane-bound organelles such as the nucleus (shown as a red sphere), mitochondria (light green), lysosome (dark blue), peroxisome (yellow), endoplasmic reticulum (yellow), and Golgi apparatus (light blue). The right half shows a schematic of membraneless organelles (blue, purple, green, and red), together with classic organelles. Membraneless organelles are distributed in both the nucleus (the paraspeckle, Cajal body, PML body, nuclear speckle, and nucleolus) and cytoplasm (the stress granule and processing body).

Known membraneless organelles in the human nucleus include the nucleolus, paraspeckles, promyelocytic leukemia (PML) bodies and Cajal bodies, and those in the cytoplasm include centrosomes and stress granules (Table 1) [1, 14, 16, 17]. Most of these cellular bodies are spherically shaped and contain viscous liquid materials [18]. These cellular bodies are generally smaller than the average size of classic membrane-bound organelles (~1  $\mu\text{m}$ ) (Table 1) [26].

Recent advances in imaging technologies have allowed researchers to determine the contents of each LLPS-mediated cellular body. These cellular bodies comprise proteins and other molecules (Table 1) [14]. Of these, nucleic acids (mostly RNAs) and RNA-binding proteins are essential to form the organelles [14]. RNA-binding proteins associated with LLPS typically harbor specific domains designated as intrinsically disordered regions (IDRs) [19]. IDRs are characterized by low-complexity sequences and are enriched with particular polar and charged amino acids [14]. IDRs do not form fixed 3-dimensional structures [27]. Interactions among IDRs of RNA-binding proteins are assumed to be the driving force of LLPS [13, 27]. IDRs, particularly those enriched with uncharged polar amino acids, are prone to self-assembling and occasionally produce irreversible protein aggregation in cells [12]. Such protein aggregation from dysregulated LLPS leads to neurodegenerative disorders (discussed later).

Recent studies have established several databases and prediction algorithms of LLPS-associated proteins. These databases include LLPSDB (<http://bio-comp.ucas.ac.cn/llpsdb> or <http://bio-comp.org.cn/llpsdb>), PhaseSepDB (<https://phasepro.elte.hu>), DrLLPS (<http://llps.biocuckoo.cn/>), RNA granuleDB (<http://rnagranuledb.lunenfeld.ca>), and HUMAN CELL MAP (<https://cell-map.org/> or <https://humancellmap.org/>), and the algorithms include UNIPROP (<https://www.uniprot.org/>), BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), ProtParam (<https://web.expasy.org/protparam>), CIDER (<http://pappulab.wustl.edu/CIDER/>), MobiDB (<http://mobidb.bio.unipd.it/about>), D2P2 (<http://d2p2.pro/>), DisMeta (<http://www-nmr.cabm.rutgers.edu/bioinformatics/disorder/>), Pi-Pi predictor (<https://cdn.elifesciences.org/articles/31486/elifesciences-31486-suppl-v1.zip>), PLAAC (<http://plaac.wi.mit.edu/>), and ZipperDB (<https://services.mbi.ucla.edu/zipperdb/>) [28, 29].

One of the well-known examples of membraneless organelles in mammalian cells is the paraspeckle. Paraspeckles were observed in a wide range of cell lines of humans and other animals [30, 31]; however, at least in mice, paraspeckles originate in a cell-type-specific manner [32]. Reportedly, paraspeckles contain more than 60 proteins including the non-POU domain containing octamer binding (NONO; alias, P54<sup>NRB</sup>), the splicing factor proline and glutamine rich, the fused in sarcoma (FUS),

and the paraspeckle component 1, together with a long noncoding RNA designated as *NEAT1* (alias, *MEM $\epsilon$ / $\beta$* ) [33]. Paraspeckles have been implicated in transcriptional regulation and the nuclear retention of hyperedited mRNAs [20]. We and other researchers have proposed that paraspeckles may be a functional platform of SRY-box transcription factor 9 (SOX9) [34, 35], a key factor for testicular development and chondrogenesis [36].

### In vitro and in vivo studies for LLPS

To date, several studies were conducted to analyze LLPS in vitro and in vivo [28, 37]. The common definition of a phase-separated structure is that it is spherical, fuses, and recovers from photo-bleaching [28]. A traditional approach to detect LLPS in vitro is direct observation of liquid-like droplets using microscopes [28]. LLPS can also be assessed through turbidity measurement and centrifugation [28, 38]. In addition, various in vitro assays, such as fluorescence recovery after photobleaching and treatment with 1,6-hexanediol, were performed to demonstrate LLPS in living cells [39]. However, the results of in vitro and in vivo assays for LLPS need to be interpreted with caution, because many proteins have potential to cause nonspecific phase separation, particularly when overexpressed or under some artificial conditions [28, 37]. The occurrence of protein assemblies is not sufficient to conclude that the proteins participate in LLPS in the context of biological function. Thus, carefully designed experiments are required to assess LLPS. In this regard, technical advance in fluorescence time-lapse microscopy and other methods will enable real-time monitoring of LLPS [40].

### Physiological Roles of LLPS

LLPS facilitates various biological processes by gathering specific sets of molecules into a subcellular space [13]. Moreover, these organelles can suppress signal transduction by sequestering signaling molecules, and can also serve as an organizational hub [27]. Consequently, these organelles contribute to various cellular reactions including transcriptional regulation, RNA processing, and heterochromatin formation [19, 21]. In particular, LLPS-mediated organelles produce the interaction among transcription factors, their coactivators, and RNA polymerase II [41]. Moreover, LLPS is known to support the function of super enhancers [13]. Accumulating evidence suggests that LLPS participates in the developmental decision of germ cells and somatic cells [15].

### Dysregulated LLPS Leading to Disorders

As mentioned, physiological LLPS is a transient and reversible phenomenon; however, dysregulated LLPS has the

**Table 1. Major cellular bodies created through liquid-liquid phase separation**

Name	Cellular location	Cell type	Number per cell	Size (nm)	Predicted function	Major components		References
						Protein	Nucleic acids or RNP	
Nucleolus	Nucleus		1	1,000-10 000	Ribosome synthesis and assembly	NOLC1, NCL	snoRNA, ribosome	[1, 16, 17]
Perinuclear compartment	Nucleus	Tumor cells	1	250-4000	Polymerase 3 RNA metabolism	KSRP, RAVER1, HNRPK	RNA of RNA polymerase 3	[1, 16, 17]
Cajal body	Nucleus		1-5	100-2000	snRNP assembly and modification	NOLC1, GAR1	snRNA, scaRNA	[1, 14, 16, 17]
Nuclear speckle	Nucleus		25-50	500-1000	Pre-mRNA splicing	SRSF4, CPSF6, SRSF2	ncRNA, snRNP	[1, 14, 16, 17]
Paraspeckle	Nucleus		5-20	500-1000	Regulation of gene expression	SFPQ, NONO, SOX9, WTI	lncRNA	[1, 14, 16, 17]
PML body	Nucleus		5-30	250-500	Control of cellular senescence and stem cell self-renewal	CREBBP, DAXX, ATRX	Nascent RNA at the periphery	[1, 14, 16, 17]
Polycomb-group proteins body	Nucleus		6-14	200-1500	Regulation of gene expression	PHC2, RING1, CTCF	DNA, RNA	[1, 16, 17]
OPT domain	Nucleus		one (or a few)	1000-3000	Response to the replication stress	MDC1, BPI, PTF8	Nascent RNA	[1, 16, 17]
Gemini of Cajal body	Nucleus		1-5	100-2000	Unknown	SMN1, GEMIN8	Unknown	[1, 16, 17]
Histone locus body	Nucleus		1-16	1000	Processing of histone pre-mRNA	SLBP, NELFE	Histone DNA, U7 snRNA	[1, 14, 16, 17]
Cleavage body	Nucleus		1-4	300-1000	RNA metabolism	TFIIF $\alpha$ , CSTF2	Unknown	[14, 16, 17]
Sam68 nuclear body	Nucleus		10-30	300-1000	mRNA trafficking	hnRNPg, YTHDC1	lnc RNA	[14, 16, 17]
Nuclear stress body	Nucleus		4-6	2000-2500	Response to stress	SAPB, TONEBP	Pre-mRNA, SatIII DNA	[14, 17]
Stress granule	Cytoplasm		10-100	100-200	Response to stress	EIF4B, YBX1, ZFP36	Nontranslating mRNA	[1, 14]
Processing body	Cytoplasm		2-20	100-300	Response to stress	TNRC6A, ZFP36, DCPIA	Nontranslating mRNA	[1, 14]
Centrosome	Cytoplasm		2	400-1400	Microtubule organizing center	CEP131, CETN1, CETN2	cnRNA	[14, 17]
Neuronal RNA granule	Cytoplasm	Neuron	10-50	150-1000	Protein synthesis in response to exogenous stimuli	KHDRBS1, CAPRIN1	mRNA, ribosome	[14, 17]
Mitochondrial RNA granule	Mitochondria		Variable	100-1000	RNA processing and biogenesis of mitochondrial ribosomes	MRM1, MTPAP	mRNA, mitochondrial ribosome	[14, 17]

Abbreviations: SatIII DNA, satellite III DNA; snRNP, small nuclear ribonucleoprotein.

potential to trigger irreversible protein aggregation leading to cellular dysfunction. Accumulation of proteinaceous aggregates (“amyloids”) in neuronal cells is a well-known cause of progressive neurodegeneration [19]. Such protein aggregation is caused by the synergic effect of aging and genetic defects of LLPS-associated proteins [12]. For example, mutations in the low complexity sequences of FUS, a key RNA-binding protein in the stress granule and the paraspeckle [2, 42], are associated with amyotrophic lateral sclerosis [2]. These FUS mutations encode aggregation-prone proteins [2, 43]. Nucleotide alterations in other LLPS-associated proteins were also identified in patients with amyotrophic lateral sclerosis [12]. Furthermore, dysregulated LLPS causes other neurodegenerative disorders, such as Parkinson disease, Huntington disease, Alzheimer’s disease, and frontotemporal dementia [12].

Dysregulated LLPS may also be associated with cancer. Nozawa et al. proposed that aberrant LLPS may contribute to the production and progression of tumors (eg, PML body disruption in acute promyelocytic leukemia) [44]. Furthermore, an abnormal morphology of membraneless organelles has been documented in tumor cells [45]. Dysregulated LLPS in tumor cells may alter nuclear events or epigenetic statuses [44]; however, the etiological relationship between LLPS and cancer needs to be confirmed in future studies.

## LLPS From the Viewpoint of Endocrinology

### Potential Roles of LLPS in Male Sex Development

To date, only a few studies have addressed the role of LLPS in endocrinology. In 2006, Hata et al. reported that SOX9 colocalizes with the paraspeckle protein NONO in the murine chondrocyte cell line ATDC5 [26]. SOX9 is a transcription factor that regulates several target genes to promote chondrocyte differentiation and testicular development [36]. Hata et al. showed that SOX9 and NONO cooperatively transactivate the promoter of the SOX9-target gene *Col2a1*. These findings suggested a link between SOX9 and LLPS. In this regard, SOX9 has previously been reported as one of the protein components of paraspeckles [14]. Thus, paraspeckles may serve as the functional platform of SOX9 in chondrocytes. In this scenario, NONO likely facilitates the activity of SOX9 in the paraspeckle.

More recently, we reported that SOX9 colocalizes with NONO in many, but not all, of immortalized Sertoli cells of mice [35]. This colocalization was also observed in a certain number of primary Sertoli cells obtained from 13-day-old mice. Furthermore, in silico analysis identified multiple IDRs, a characteristic feature of LLPS-associated proteins [46], in murine and human SOX9 [35]. These

results indicate that paraspeckles contribute to the function of SOX9 not only in chondrocytes, but also in Sertoli cells. Considering that SOX9 dysfunction results in 46,XY disorders of sex development (DSDs) [47, 48], LLPS is likely to be indispensable for testicular development. The association between the paraspeckle and male sex development is further supported by the studies of Kuwahara and Dong et al., which showed that 2 paraspeckle proteins (NONO and paraspeckle component 1) are expressed in murine Sertoli cells and participate in the androgen receptor (AR)-mediated transcription [49, 50].

LLPS may also be associated with other molecules involved in male sex development. First, the polycomb protein chromobox 2 (CBX2), a chromatin modulator involved in testicular development [51–53], has recently been linked to LLPS [41, 54]. Reportedly, CBX2 contains an IDR and assembles polycomb group condensates in membraneless organelles [41]. It appears that CBX2 uses LLPS to connect to the target sequences in the genome. Thus, 46,XY DSDs in the previously reported patient with a CBX2 variant [53] may result from perturbed LLPS in the developing testis. Second, we have previously reported that the mastermind-like domain containing 1 (MAMLD1) exhibits a speckled-type distribution in the nucleus of cultured mouse Leydig tumor cells [55]. The distribution pattern is indicative of LLPS. These data support the association between LLPS and male sex development because MAMLD1 has been implicated in testicular steroidogenesis [56]. Of note, we found that 2 protein-truncating *MAMLD1* variants identified in patients with 46,XY DSDs were incapable of localizing to nuclear speckles [55]. Thus, MAMLD1 likely functions in specific LLPS-mediated nuclear organelles of testicular cells. Last, AR may also reside within a LLPS-mediated cellular body. This notion is based on the observation that poly-glutamine expansions within AR result in a progressive neurodegenerative disorder [57]. However, further studies are necessary to examine whether CBX2, MAMLD1, and AR actually reside within LLPS-mediated organelles in human cells.

### Dysregulated LLPS in Type 2 Diabetes

Although dysregulated LLPS primarily results in neurodegenerative disorders, it can also underlie other diseases. In particular, aberrant LLPS has been associated with type 2 diabetes [58]. It was reported that the intracellular accumulation of misfolded islet amyloid polypeptide (IAPP) results in pancreatic  $\beta$ -cell dysfunction and resultant type 2 diabetes [59]. Pytowski et al. proposed that such protein aggregation in  $\beta$ -cells is caused by dysregulated LLPS [58]. Interestingly, Horvath et al. have documented that pro-IAPP crosstalks with



$\alpha$ -synuclein, a protein that forms amyloids in patients with Parkinson disease, and accelerates its aggregation [59]. Thus, dysregulated LLPS is likely to be associated with both type 2 diabetes and Parkinson disease. These findings may explain why patients with type 2 diabetes have a relatively high risk of Parkinson disease.

### Other Potential Roles of LLPS in Endocrine Cells

It is now apparent that LLPS-mediated cellular bodies are present in all living human cells. Because LLPS can dynamically respond to extracellular and intracellular stimuli [31, 60], this phenomenon is optimal for the fine-tuning of hormone secretion. Indeed, Chaoui et al. reported that SOX10, a transcription factor involved in neural crest cell development [61], colocalizes with NONO in HeLa and SK-MEL-5 cells [62]. Thus, SOX10 may function in paraspeckles, as in the case of SOX9. Because SOX10 is known as one of the causative genes of Kallmann syndrome [63], this suggests the possible link between LLPS and gonadotropin secretion. In addition, Nair et al. reported that LLPS likely mediates estradiol-dependent enhancer activation in human breast cancer cells [64]. Actually, LLPS-mediated cellular bodies, either alone or together with classic membrane-bound organelles, may have hitherto unrecognized roles in hormone secretory cells. Further studies are needed to clarify the significance of LLPS in hormone secretion. In addition, it is also necessary to determine whether dysregulated LLPS is associated with endocrine disorders other than type 2 diabetes. For example, the contribution of defective LLPS to autoimmune endocrine disorders remains unknown, although LLPS has been implicated in immune reactions in the body [65].

### Conclusions

Recent studies have revealed that every living eukaryotic cell contains a number of membraneless organelles created via LLPS. LLPS likely contributes to male sex development by providing a functional platform for SOX9 and CBX2 in testicular cells. On the other hand, dysregulated LLPS in pancreatic  $\beta$ -cells is assumed to underlie type 2 diabetes through deleterious IAPP aggregation. Still, we are just beginning to understand the physiological and pathogenic roles of LLPS in endocrine cells.

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### Additional Information

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**Data Availability:** Data sharing is not applicable to this article because no datasets were generated or analyzed during the current study.

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